Originals

Molecular cloning of a group of mouse pancreatic islet beta-cell-related genes by random cDNA sequencing

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Summary To understand the molecular basis of glucose concentration-responsive insulin synthesis and secretion from pancreatic islet beta cells, a group of pancreatic islet beta-cell-related cDNAs was cloned. A pair of cDNA libraries was constructed from a mouse pancreatic islet beta-cell line of MIN6, which was cultured in either high glucose or low glucose media. By applying a random cDNA sequencing approach, 503 and 395 independent species were obtained from a total of 1,011 and 762 clones in the high glucose and low glucose library, respectively. The unknown genes comprised the majority of about 70 % independent clones in both libraries. In Northern blot analysis, 311 (69.4 %) of 448 independent

clones showed positive signals within 72 h of autoradiographic exposure. Surprisingly, 150 (48.2 %) out of 311 positive clones showed positive signals to MIN6 cells, but not to NIH/3T3 fibroblasts. The expression level of three unknown clones were glucose-concentration dependent. Combination of a random cDNA sequencing approach and Northern blot analysis is useful to obtain a large number of novel genes and islet beta-cell-related genes. [Diabetologia (1995) 38: 381–386]

Key words Random cDNA sequencing, MIN6, pancreatic islet beta cell.

Understanding the molecular basis of pancreatic islet beta cells is essential in order to develop a logical treatment for diabetes. In addition to glucokinase [1–3] and mitochondrial genes [4–5], which are known to be aetiological for non-insulin-dependent diabetes or maturity-onset diabetes of the young, there are others which are related to islet beta-cell

function and diabetes. It is speculated that the highly-differentiated functions of islet beta cells are based on a group of cell-specific genes. Recently, large-scale cDNA analysis has become a valuable way to examine and register expressed genes in various tissues by determining expressed sequence tags [6–9]. Abundant transcripts, which are frequently encountered in random sequencing, are assumed to play relatively more important roles than rare transcripts with exceptions including transacting factors and rate-limiting enzymes among others. With this in mind we randomly sequenced cDNA to clone unknown genes which are abundantly expressed and are presumably important for islet beta-cell functions. In this study we report the acquisition of a group of islet beta-cell-related genes by combining a random cDNA sequencing of a total of 1,773 clones from a mouse islet beta-cell line of MIN6 [10] with Northern blot analysis of 448 clones.

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; AGPC, acid guanidinium thiocyanate-phenol-chloroform; bp, base pair(s); IAPP, islet amyloid polypeptide; HUMMHA 123, human MHC protein homologous to chicken B complex protein; PACAP, pituitary adenylate cyclase activating polypeptide precursor.

Materials and methods

Cell preparation. A mouse islet beta-cell line of MIN6 (passage 17–20), established from a transgenic mouse harbouring an insulin promoter plus Simian mouse virus (SV)40 T-antigen fusion construct, was cultured up to 70–80% confluency in Dulbecco's modified Eagle's medium (DMEM) containing 25 mmol/l glucose and 10% fetal calf serum (FCS). Cells were then cultured in 10% FCS and DMEM for 12 h under either high glucose (25 mmol/l) or low glucose (0.5 mmol/l) conditions.

cDNA library construction. RNA was extracted from MIN6 cells by the acid guanidinium thiocyanate-phenol-chloroform (AGPC) method [11]. Five micrograms of poly(A)⁺ RNA was incubated with 200 pmol of *Xho*I-(dT)₁₅ primer (ATTAACCTCAGCCACTCGAGTTTTTTTTTTTTTTT), 1 mmol/l 2-mercaptoethanol, 500 µmol/l each of dNTPs, 200 U of RNasin (Promega Madison, WI, USA), 100 U of Avian myeloblastosis virus (AMV) reverse transcriptase (Seikagaku Corporation Chuo-ku, Tokyo, Japan), 50 mmol/l Tris-HCl (pH 8.5), 8 mmol/l MgCl₂, 30 mmol/l KCl at 37°C for 1 h. It was then incubated with 50 U of *Escherichia coli* DNA polymerase I, 3 U of RNase H, 500 µmol/l each of dNTPs, 50 mmol/l Tris-HCl (pH 7.5), 10 mmol/l MgCl₂, 1 mmol/l 2-mercaptoethanol, and 90 mmol/l KCl at 14°C for 1 h, then at 22°C for 1 h. It was incubated with 10 U of T4 DNA polymerase at 37°C for 10 min. The product was eluted by centrifugation with a Chroma spin column S-400 (Clontech Palo Alto, CA, USA) at 700 × g for 5 min.

An *Eco*RI adaptor was ligated to cDNA following the recommendations of Takara (Otsu, Shiga, Japan). After digestion with *Xho*I, the product was eluted with a Chroma spin column. The 5' end of the cDNA was phosphorylated with 10 U of T4 polynucleotide kinase and 100 µmol/l of ATP at 37°C for 30 min. One microgram of λ-ZAPII arms (Stratagene, La Jolla, CA, USA) double-digested with *Xho*I and *Eco*RI was ligated to the cDNA using a Takara ligation kit. Phage packaging was done with the Gigapack II Gold (Stratagene), following the protocol recommended by the manufacturer.

Converting phage clones en masse to pBluescripts. The *E. coli* strain XL1-Blue infected with about 5,000 phage clones were incubated in Luria-Bertari (LB) top agar at 37°C overnight. Eluted λ-ZAPII phages were converted *en masse* to pBluescript SK (–), using R408 helper phage, following the protocol recommended by Stratagene.

Random single pass sequencing. Randomly selected colonies were incubated in LB medium with 50 µg/ml of ampicillin at 37°C overnight. The pBluescript DNA was extracted using the alkaline lysis method. Sequencing of the inserts was performed with ABI 373A autosequencer (Applied Biosystems, Foster, CA, USA) with a dye-labelled T7 or T3 primer, following the manufacturer's protocol.

Northern blot analysis. RNA was extracted by the AGPC method from MIN6 cells cultured in high or low glucose media, NIH/3T3 fibroblasts, and organs of C57BL/6 mice including brain, liver, spleen, kidney, and pancreas. Twelve micrograms each of total RNA was electrophoresed through a 1% denaturing agarose gel and was transferred to Hybond-N (Amersham, Buckinghamshire, England). To make probes, each diluted pBluescript insert was amplified by polymerase chain reaction (PCR) at 94°C for 1 min, 50°C for 1 min, 72°C for 2 min, after 40 cycles with 30 pmol of SK and T7 primers. Free nucleotides were removed using glass powder. The labelling by α-³²P dCTP was performed by the multiprime labelling system (Amersham). The hybridization was performed in 50% formamide, 5 × SSPE buffer, 5 × Denhardt's reagent, and 0.5% SDS at 42°C overnight. Membrane was washed three

Fig. 1 A, B. Relationship between known and unknown genes, and between frequencies of appearance and the number of species. **(A)** In the high glucose library, a total of 1,011 clones were computer-searched, and 503 independent species were obtained, among which 143 (28.4%) and 360 (71.6%) species were known and unknown genes, respectively. Relationship between frequencies of appearance and the number of species of known (■) and unknown genes (□) is shown. Genes with frequencies of more than ten times (> 10) were 6 and 1 species in the known and unknown genes, respectively. **(B)** In the low glucose library, 97 (24.6%) and 298 (75.4%) species were known and unknown, respectively. The number of known and unknown species decreased along with the increasing frequencies of appearance in both libraries

times with 1 × SSPE and 0.1% SDS at 65°C for 30 min, and exposed for autoradiography on Kodak XAR film (Rochester, New York, USA) for 2–72 h at –80°C.

Results

Analysis of randomly-sequenced cDNA. A pair of cDNA libraries was separately constructed from a mouse MIN6 pancreatic islet beta-cell line, which was cultured in high or low glucose media. The sizes of inserts of randomly-selected clones ranged from zero to about 2,000 base pairs (bp) with an average of 800 bp. The randomly-selected clones from the high glucose and low glucose library were directionally sequenced from the 3'-end with a dye-labelled T7 primer, using a fluorescence-based DNA autosequencer. Only when cDNA sequences were not readable from the 3'-end due to a long poly(A) tail, they were sequenced with a dye-labelled T3 primer from the 5'-end. The average length of compiled sequences of inserts was 252 bp. The clones with inserts of less than 60 bp were eliminated, because the longer sequence supplies more information about its identity than a fragmentary cDNA. Finally, a total of 1,011 and 762 clones were sequenced from the high glucose and low glucose library, respectively. The sequence similarities of 1,773 clones were first

Table 1. Summary of known genes in random sequencing. Frequencies of appearance of representative known genes in the random sequencing are listed

Genes	Frequency		Genes	Frequency	
	High glucose	Low glucose		High glucose	Low glucose
<i>Secretory protein (14 species)</i>			<i>Protein synthesis (42 species)</i>		
Insulin II	45	91	Acidic ribosomal phosphoprotein PO	6	5
Insulin I	21	17	Elongation factor 1- α	6	2
IAPP	20	6	Ribosomal protein S14	4	2
Chromogranin A	19	7	Ribosomal protein S8	3	4
Cholecystkinin	6	3	Ribosomal protein L27	2	4
Prealbumin	5	2			
Secretogranin II	3	4	<i>Cytoplasm and others (109 species)</i>		
PACAP	1	3	Mitochondrial genome	47	32
Dynorphin	2	0	Ribophorin I	10	0
Neuroleukin	1	0	19.5 kD protein	8	2
.....			Ubiquitin	7	1
<i>Cytoskeleton (5 species)</i>			Cystatin C	0	7
α -Tubulin	3	0	Brain calbindin-d28k	6	0
Cofilin	2	0	Insulinoma rig	2	4
β -Tubulin	1	1	Ferritin light chain	5	0
.....			Tryptophan hydroxylase	4	0
<i>Membrane protein (16 species)</i>			Aldolase A	0	4
HUMMHBA123	11	0	Thexokinase	3	0
Laminin receptor	7	1	Stathmin	3	0
Basigin	6	0	Heat shock cognate hsc73	1	2
Tyrosine protein kinase B	5	0	Nucleoside diphosphate kinase	1	2
MHC class I H2-TL-27-129	2	0	Creatine kinase B	1	2
E-cadherin	1	0	Cu-Zn superoxide dismutase	1	2
MHC class I H-2D-k	0	1	Cyclophilin	2	0
.....			Calregulin	2	0
<i>Nuclear protein (8 species)</i>			Calreticulin	1	1
Homeobox Hox-7.1	4	0	Glyceraldehyde 3-phosphate dehydrogenase	1	1
HMG-17 chromosomal protein	2	0			
Nucleolar protein N038	2	0			
.....					

compared with each other and then with the DNA data base in GenBank Bethesda, MD, USA (Release 80.0 December, 1993) encompassing mammalian genes, using a FASTA program [12, 13]. Genes with more than a 90 % similarity with the known genes in GenBank were defined as known, while others were defined as unknown. We obtained 503 and 395 independent species in the high glucose and low glucose library, respectively. Known genes comprised the minority of 143 (28.4 %) and 97 clones (24.6 %) in the high glucose and low glucose library, respectively (Fig. 1).

Most known and unknown genes appeared once and the number of known and unknown genes exponentially decreased along with the increasing frequency of appearance in both libraries (Fig. 1). The genes with an appearance rate of more than 1 % were numbers 7 and 4 in the high glucose and low glucose library, respectively. Appearance rates for insulin I [14] of 2.1 % and mitochondrial genome [15] of 4.6 % in the high glucose library were comparable to those of 2.2 and 4.2 % in the low glucose library, while those for insulin II of 4.5 %, islet amyloid polypeptide (IAPP) [16] of 2.0 %, chromogranin A of

1.9 %, and human MHC protein homologous to chicken B complex protein (HUMMHBA123) of 1.1 % in the high glucose library were apparently different from 11.9, 0.8, 0.9, and 0 % in the low glucose library, respectively. Clones of insulin II, insulin I, and IAPP had poly(A) tails of different lengths ranging from 0 to about 120 daltons, which suggested the different origins of each clone. On the other hand, chromogranin A and HUMMHBA123 had no poly(A) tails, which suggested the presence of *Xho*I site in the sequence.

We categorized known genes to 'secretory protein', 'cytoskeleton', 'membrane protein', 'nuclear protein', 'protein synthesis', 'cytoplasm and others', although most of their precise functions are unknown (Table 1). In 'secretory protein', cholecystkinin, prealbumin, secretogranin II, and pituitary adenylate cyclase activating polypeptide precursor (PACAP) in addition to insulin, IAPP, and chromogranin A were found in both libraries. The protein-synthesis-related genes were composed of as many as 42 species. There were another 109 species including proteasome, ATPases, kinases, etc., and those with unknown functions. Only 60 species (15.2 %) of the

Fig. 2. Northern blot analysis to obtain MIN6-related clones. We hybridized 448 independent clones with 12 µg of total RNA of MIN6 cells, which were cultured under low or high glucose conditions and that of NIH/3T3 fibroblasts, as abbreviated, L, H, and F, respectively. Exposure was for 2–72 h. Top and second rows, eight representative known genes commonly expressed in MIN6 cells and NIH/3T3 fibroblasts, and those expressed in only MIN6 cells are shown, respectively. On the third and bottom rows, the unknown clones commonly expressed in both cell lines, and those expressed in only MIN6 cells are shown, respectively

low glucose library overlapped those of the high glucose library.

Northern blot analysis to explore cell-specific and glucose concentration-responsive expression. For the first screening, gene expression and sizes of transcripts were examined by Northern blot analysis in regard to the specificity to MIN6 cells or NIH/3T3 fibroblasts, and regarding responsiveness to the high glucose or low glucose metabolic conditions. A total of 448 independent clones including 110 known and 204 unknown clones in the high glucose library, and 17 known and 117 unknown clones in the low glucose library, were used as ³²P labelled probes. A total of 311 probes (69.4%) showed positive signals within 72 h of autographic exposure. A total of 150 (48.2%) clones including 43 known and 107 unknown genes hybridized to MIN6 transcripts but not to those of NIH/3T3 fibroblasts. A total of 158 (50.8%) clones including 63 known and 95 unknown genes hybridized to both MIN6 and NIH/3T3 transcripts. Three other clones showed positive signals to NIH/3T3 transcripts, but not to those of MIN6. Most positive genes in MIN6 hybridized to both high glucose and low glucose transcripts at the same intensity. Three unknown genes were glucose concentration-responsive. The HG14-61 found in the high glucose library showed a stronger signal to high glucose transcripts, while LG4-87 and LG5-25 found in the low glucose library showed stronger signals to low glucose transcripts, compared with other known genes used as control (Fig. 2).

To confirm the organ specificity, the clones, which showed a positive signal to MIN6 cells, but not to NIH/3T3 fibroblasts, were further analysed using

RNA extracted from various organs from C57BL/6 mice including brain, liver, spleen, kidney, and pancreas. Eleven of 16 clones so far examined showed a weak signal to pancreatic RNA, but did not show any signals to RNA of other organs (Fig. 3).

Discussion

To obtain pancreatic islet beta-cell-specific genes and glucose concentration-responsive genes, we constructed a pair of MIN6 cDNA libraries which were cultured in high glucose or low glucose media. MIN6 (passage 17–20) secreted insulin about 3, 4 and 6 times more in 5, 15, 25 mmol/l than in 0.5 mmol/l glucose for 12 h, respectively (data not shown). We assumed that some controlling genes which were involved in insulin synthesis and secretion during the relatively early stage, could be obtained by comparing cDNA from MIN6 incubated for 12 h in 0.5 and 25 mmol/l glucose.

Our random cDNA sequencing firstly enabled us to observe the genetic pattern of expressed genes in islet beta cells. A mammalian cell is assumed to express genes of as many as 15,000–30,000 species out of a presumed total of 50,000–100,000 genes. In our random sequencing of 1,773 clones, the majority of cDNA clones appeared once and the number of species decreased exponentially along with the increasing frequency of appearance. This is consistent with the concept that a great variety of genes are expressed in relatively low abundance in vivo, and it showed that our libraries included enough species of gene for random cDNA sequencing.

Fig. 3. (A–F) Gene expression in various organs. Unknown genes, which showed positive signals only to MIN6 cells but not to NIH/3T3 fibroblasts, were examined regarding organ specificity by Northern blot analysis. (A) Twelve micrograms each of total RNA was electrophoresed through a 1% denaturing agarose gel. (B)–(D) Eleven of 16 clones showed weak positive signals to RNA of the pancreas but not to those of other organs. For example, HG2-57, HG3-72 and HG10-84 showed strong signals to MIN6, weak positive signals to the pancreas, but not to other organs at 72 h of autoradiographic exposure. (E) LG3-12 showed a positive signal to all of the organs at 6 h of exposure. (F) Insulin II showed a signal to the pancreas at 6 h. H, B, L, S, K, and P stand for MIN6 cells cultured under high glucose conditions, brain, liver, spleen, kidney, and pancreas of C57BL/6 mice, respectively

Insulin is one of the most important genes in islet beta cells, and the appearance rate of insulin II was actually the highest in the libraries except for mitochondrial genes, which comprised many genes including tRNA, rRNA, ATPase, among others. Appearance rates in the random sequencing should generally reflect abundance of transcripts *in vivo*, but the reflection is not perfect. Appearance rates of insulin II, chromogranin A and IAPP among others in the high glucose library were apparently different from those in the low glucose library. Because signals of those transcripts in Northern blot analysis were comparable between high glucose and low glucose conditions the differences in the appearance rate may be within the statistically insignificant error range. Our goal is not such a precise estimation of transcripts, but to obtain islet beta-cell-specific and glucose-concentration-responsive genes. Only 60 clones (15.2%) from the low glucose library overlapped those of the high glucose library. This, however, does not suggest a large difference in the transcript species between low glucose and high glucose conditions. The number of common genes should increase, as the number of clones in random sequencing is increased.

Recently, random cDNA sequencing of human pancreatic islets containing a mixture of cells was reported [17]. Only 17 (9.9%) of the 172 known genes reported overlapped those of ours. The 9.9% value is reasonable, considering the difference between the libraries; ours with inserts of more than 400 bp from a pure mouse islet beta-cell line and the reported library with inserts of more than 1,000 bp from various human islet cells, and considering the similar proportion of 15.2% overlapped genes between our high glucose and low glucose libraries.

Secondly, cell- and metabolic condition-related genes were extracted using extensive Northern blot analysis. To our surprise, 150 (48.2%) species of 311 positive genes only showed positive signals to MIN6 cells, but not to NIH/3T3 fibroblasts. These were candidate genes specifically expressed in islet beta cells. The generally accepted idea that transcripts specific for a particular cell are relatively small in number and that most transcripts are of house-keeping genes, was not the case for islet beta cells. Rather, islet beta cells with a highly differentiated ability to secrete insulin are regarded as being based on a large number of cell-specific genes. Because the majority of clones, which showed positive signals to MIN6 cells but not to NIH/3T3 fibroblasts at the first screening, showed weak positive signals to transcripts of pancreas but not to those of other organs, they were likely to be specifically expressed in islet beta cells *in vivo*. Because islet beta cells comprise a small percentage of the pancreas, it is reasonable that signals from islet beta-cell-specific genes are weak to the RNA extracted from the whole pancreas. In Northern blotting, there were only three glucose concentration-responsive genes, but insulin was not included among them. It has been suggested that insulin is regulated by translational rather than transcriptional control.

To obtain cell-specific clones, many subtraction procedures have been used [18–20]. Because one in every two or three clones was a candidate gene specific to islet beta cells in our random cDNA sequencing approach, one does not need a subtraction to obtain islet beta-cell specific genes.

By the combination of a random cDNA sequencing approach and Northern blot analysis using fibroblasts as a control, a large number of unknown pancreatic beta-cell-related clones were obtained from a cultured islet beta-cell line. Although the final characterization has not been completed, our exhaustive efforts to obtain cell-specific and metabolic condition-responsive genes supplied us with a large number of candidate genes. To explore the significance of these genes in islet beta-cell function and diabetes, Northern blot analysis including other neuroendocrine cells, *in situ* hybridization, and functional analysis are underway in addition to complete sequencing.

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